Refractoriness of KB Cell Cultures Carrying Japanese B Encephalitis Virus to Encephalomyocarditis Virus Infection* (34017)

EIICHI FURUSAWA, SHINOBU FURUSAWA, AND WINDSOR CUTTING

Department of Pharmacology, University of Hawaii School of Medicine, Honolulu, Hawaii 98816

To supplement antiviral screening in mice, we use infections in KB cell lines with the following viruses: encephalomyocarditis (EM C), lymphocytic choriomeningitis (LCM), Japanese B encephalitis (JBE), vesicular stomatitis (VS), polio types I, II, Reo type II, vacinia, adenotypes 12 and 18. While most of these viruses show complete cytopathic effects (CPE), with LCM and JBE only some of the cells are destroyed. In this paper, we report that the cells not damaged by JBE virus grow continuously and have acquired a virus-carrier state. Also, JBE-carrying cells are highly resistant to superinfection with EMC virus. The refractory state to EMC seems not to be induced by cellular interferon but to be related to a viral genome characteristic referred to as of intrinsic interference by Marcus and Carver (1).

Materials and Methods. 1. *Viruses.* JBE virus (Nakayama strain, mouse brain passaged, ATCC no. VR74) was supplied from Dr. L. Rosen, Pacific Section, National Institute of Allergy and Infectious Diseases, NIH. The VS, polio type II (Lansing strain), and Reo type II (strain D5) were supplied from Dr. P. Loh, Dept. of Microbiol., Univ. of Hawaii, from material passaged in HeLa cells. The LCM (NY 621 strain, mouse brain passaged), EMC, polio type I (Mahoney strain), vaccinia (IHD strain), adeno types 12 and 18 were standard strains from our laboratory. All the viruses except JBE and LCM had been passaged for over 10 generations in KB cells before use.

2. *KB cells.* The line was supplied from Dr. Rosen, and had been cultured in medium 199 with 2% calf serum for several years.

3. *Virus assay.* Mice were as follows: 6–7 g mice for JBE by intracerebral (ic) route; KB cells for other viruses. Each 10-fold dilution was inoculated into 3 mice or 3 culture tubes, and the LD_{50} or $TCID_{50}$ was calculated according to the method of Reed and Muench (2). Some viruses were titrated in 2 tubes/each 10-fold dilution and expressed the titers as $TCID_{100}$.

Results. 1. Establishment of a KB cell line carrying JBE. About 5×10^6 LD_{50}/0.1 ml of JBE virus (10% mouse brain extract) was inoculated onto KB cell monolayers (5×10^4 cells) in 1 ml of medium. The infected cultures were twice washed with Hanks solution 2-hr postinfection to remove the cycotoxic effect of mouse brain extract. Medium was changed every 2 days. The CPE of the virus appeared on day 5 and reached 2+ degree (25–50% cell damage) on day 7, but was not progressive and finally the damaged area was completely repaired by the growth from neighbor cells. Thereafter, the culture cells were continuously subcultured every 7–10 days for 11 months. The growth rate and the morphological figures of the cells are not different from the noninfected normal KB cells. The infectivity of the culture medium has

TABLE I. Continuous Liberation of Mature JBE Virus from JBE-Carrying KB Cell Cultures into the Medium.[a,b]

3	6	10	15	20	25	30	35	40	generation
3.5	3.8	3.5	3.5	3.2	3.8	3.2	3.5	3.2	virus titer

[a] The culture medium on days 4, 5, or 6 after subcultures were titrated in mice.

[b] No. of generations of JBE-carrying cells and virus titer in the medium (log LD_{50}/0.03 ml).

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TABLE II. EMC-Refractory State of JBE-Carrying KB Cell Cultures.[a]

Multiplicity of infection ($TCID_{50}$/cell)	Incubation period (days)															
	1			2				5		8		15		20		
	JBE	Normal		JBE		Normal		JBE		JBE		JBE		JBE		
	CPE	CPE	TCID	CPE	TCID	CPE	TCID	CPE	TCID	CPE	TCID	CPE	TCID	CPE	TCID	
1000	2+	4+		2+	3			2+	3	1+	2	0	0	0	0	
100	1+	4+		1+	3			1+	3	0	1	0	0	0	0	
10	0	4+	7	0	1			0	0	0	0	0	0	0	0	
1	0	2+	5	0	0	4+	7	0	0	0	0	0	0	0	0	
0.1	0	1+	5	0	0	4+	7	0		0		0		0		
0.01	0	0		0		4+		0		0						
0.001	0	0		0		4+		0		0						
0.0001	0	0		0		4+	7	0		0						

[a] Various multiplicities of EMC virus were inoculated into JBE-carrying and normal KB cells. Two hr later, the cells were washed with Hanks solution to remove unadsorbed virus. Culture medium was changed every 2 days. The media of some tubes were collected for titration of the infectivity ($TCID_{100}$/0.1 ml).

been periodically titrated in mice, as shown in Table I. The stable liberation of mature virus into the medium without any CPE indicates that the infected cell cultures have become carriers of JBE.

2. EMC-refractory state of KB cell cultures carrying JBE. It was found that the KB cell cultures carrying JBE were completely resistant to the accidental infection of 100 $TCID_{100}$ of EMC virus which has a severe CPE on normal KB cells. Then, purposely superinfection with various multiplicities of EMC virus was tried. Table II shows the results. At a level of multiplicity of infection (m.i.) of 10 or less, JBE-carrying cell cultures were completely resistant to both CPE and virus growth, and at higher m.i. partial CPE with low grade of virus growth was seen. The virus titer was never raised over 10^3 $TCID_{100}$ in JBE-carrying cultures with partial CPE, as compared with 10^5 $TCID_{100}$ of virus growth in normal cells at an early stage of CPE (1+ or 2+) before reaching the maximum CPE (4+). Therefore, it is suggested that incomplete virus growth, which induced cell damage but little mature virus, occurred in the JBE-carrying cultures when they were infected with more than 100 m.i. of EMC virus. The remaining cells which escaped the CPE of EMC virus grew well, with repair of the damaged area of the monolayer and finally no trace of infective EMC virus could be recovered. The EMC-infected JBE-carrying cultures could be continuously subcultured with normal growth rate.

3. Infectivity of individual cells in JBE-carrying cultures. The complete refractoriness of JBE-carrying cell populations against 10 multiplicities of infection with EMC virus, which are enough to infect most of the cells at the same time, suggested that the individual cells are carrying the JBE genome although little virus (about 1 LD_{50} /10 cells) was detected in the medium. Therefore, it was of interest to know that whether the individual cells could become infective centers when they were mixed with normal cell populations. JBE-carrying cultures were trypsinized, washed (4 times with Hanks), di-

TABLE III. Infectivity of Individual Cells in JBE-Carrying Cultures.[a]

	No. of cells of JBE-carrying cultures mixed with normal cells per tube					
	75	15	3	0.6	0.12	0
No. of tubes refractory to EMC/total tubes	10/10	10/10	8/10	3/10	1/10	0/10
No. of tubes with growth of JBE virus/total tubes	ND[b]	10/10	8/10	3/10	1/10	0/10

[a] Each tube contains 0–75 cells of JBE-carrying culture mixed with 5×10^4 normal KB cells in 1.5 ml of medium. The tubes were cultured for 6 days. After harvesting each medium for detection of JBE virus propagation, the tubes were infected with 100 $TCID_{100}$ of EMC virus. Tubes showing only partial CPE were designated as in refractory state after 2-days incubation. The growth of JBE virus was confirmed by inoculation of each harvest (0.03 ml of 1:10 dilution) into mice.

[b] Not done.

luted, and suspended in 1 ml of medium at with various numbers of cells (0.12–75 cells/ml by 5-fold dilution); then to each tube was added a fixed number of normal KB cells (5×10^4 cells in 0.5 ml). The mixtures were incubated in stationary culture for 6 days. For the detection of virus in mice the medium was collected, and 100 $TCID_{100}$ of EMC virus was added to the tubes to detect the appearance of refractoriness. Table III shows the results. It was found that the refractory and infective state was related to the number of cells used: 30 cells affected 8 tubes out of 10 tubes; 6 cells, 3 tubes; and 1.2 cells, 1 tube. From this evidence and with consideration of plating efficiency (Poisson distribution of the cells), it is suggested that most of the individual cells have the JBE-genome for the refractory state and reproduction of virus.

4. No detection of interferon in KB cell lines carrying JBE. The medium of JBE-carrying cultures has the capacity to induce the EMC-refractory state in normal KB cells. Preliminary experiments indicated that this capacity was parallel to the amount of mature virus in the medium. UV irradiation, which inactivated the infectivity of medium containing virus to 1 LD_{30}, reduced the refractoriness inducing capacity almost none. Therefore, it seemed that active virus was required to induce the refractory state. It was important to know whether interferon was involved in the refractory state. Interferon was made by the method of Desmyter *et al.* (4). The JBE-carrying cultures and medium were three times frozen and thawed, and the supernatant after centrifugation (8000 rpm, 20 min) was adjusted to pH 2.0, kept at 4° for 5 days, and then returned to pH 7.0 and ultracentrifuged (50,000 rpm, 2 hr). The supernatant, as an interferon preparation, was added to normal KB cells 1 and 20 hr before virus inoculation of 10 $TCID_{100}$ of EMC, polio type II, VS, or vaccinia IHD. The results showed that no trace of interferon activity could be detected for any of the viruses tested.

5. No effect of dactinomycin on the EMC-refractory state of JBE-carrying cultures. Interferen production is inhibited by dactinomycin which inhibits the synthesis of cellular messenger RNA essential to synthesis of interferon (5). Dactinomycin (0.1 μg/ml/tube), which dose was enough to inhibit vaccinia virus propagation in HeLa cells (6), was added to the KB cell cultures, both normal and JBE-carrying cultures, 1 hr before inoculation of virus. Table IV shows the results. It was found that JBE-carrying cultures were still refractory to infection with EMC virus in the presence of the amount of dactinomycin that was inhibitory to the growth of vaccinia virus in both normal and JBE-carrying cultures. That is, the EMC-refractory state of JBE-carrying cultures seems not to involve an interferon mechanism which dactinomycin would inhibit.

6. Viral specificity of the refractory state of JBE-carrying cultures. According to the report of Marcus and Carver (1), one of the

TABLE IV. Dactinomycin-Resistant Interference State of JBE-Carrying Cell Cultures.[a]

Challenge virus	Multiplicity of infection	Incubation (days)	Dactinomycin-treated cells (CPE)		Control cells (CPE)	
			JBE-carrying	Normal	JBE-carrying	Normal
EMC	100	1	1+	4+	1+	4+
	10	1	0	4+	0	4+
	1	2	0	4+	0	4+
Vaccinia	10	2	1+	1+	4+	4+
	1	4	1+	1+	4+	4+

[a] Dactinomycin (0.1 μg/ml of medium/tube) was added 1 hr before virus inoculation. Two hr after infection, cells were washed 3 times with Hanks solution and fresh medium with the drug added.

characteristics of "intrinsic interference" (noninterferon-mediated interference or viral genome-induced cellular state of resistance) is that the cells refractory to a virus still are susceptible to a broad spectrum of other viruses, similarly tested at high multiplicity. A comparison of virus infectivity titrations using normal and JBE-carrying cell lines was done. Table V shows the results. The JBE-carrying cultures were found to have the same susceptibility as normal cells to superinfection with polio I, II, VS, Reo II, vaccinia, and adeno types 12 and 18 viruses at high multiplicity, but have some resistance to polio I, II, and VS viruses at low multiplicity. Specially, the completion of the CPE of poliovirus was delayed for 2 days at the highest dilution and VS titer was one log lower in JBE-carrying cultures than in normal cells.

7. Interference between EMC and VS viruses in the JBE-carrying cultures. As men-

TABLE V. Susceptibility of JBE-Carrying KB Cell Cultures to Superinfection of Several Viruses.

Virus		Virus dilution (10 log); and days to reach maximum CPE								
		0	−1	−2	−3	−4	−5	−6	−7	−8
Polio I and II	Normal KB	1	1	1	2	2	2	2	2	No[a]
	JBE-carrying	1	1	1	2	2	3	5	5	
VS	Normal KB	1	1	1	2	2	2	2	3	No
	JBE-carrying	1	1	1	2	3	4	5	No	No
Reo II	Normal KB	3	5	7	9	11	13	16	No	
	JBE-carrying	3	5	7	9	10	12	14	No	
Vaccinia	Normal KB	2	4	5	6	7	7	No		
	JBE-carrying	2	4	5	7	7	7	No		
Adeno 12	Normal KB	3	5	7	9	No				
	JBE-carrying	3	5	7	9	No				
Adeno 18	Normal KB	3	5	7	9	No				
	JBE-carrying	3	5	6	8	No				
EMC	Normal KB	1	1	1	2	2	2	2	2	No
	JBE-carrying	p[b]	p	No	No	No	No	No	No	No

[a] No CPE detectable within an additional 5 days of observation.

[b] Partial, nonprogressive CPE (up to 2+ degree) finally repaired by the growth of neighbor cells.

TABLE VI. Susceptibility of EMC-Infected JBE-Carrying KB Cell Cultures to Superinfection of Several Viruses.

Challenge virus	Infectivity titers ($TCID_{100}$) using: EMC-infected[a] (4 hr before) JBE-carrying cult.	EMC-infected[a] (20 days before) JBE-carrying cult.	JBE-carrying KB cell cultures	Normal KB cells
Polio I	10^7	10^7	10^7	10^7
VS	10^4	10^6	10^6	10^7
Reo II	10^6	10^6	10^6	10^6
Vaccinia	10^5	10^5	10^5	10^5
Adeno 18	10^3	10^3	10^3	10^3
EMC	0	0	0	10^7

[a] The JBE-carrying KB cultures had been infected with 100 multiplicity of EMC virus 4 hr or 20 days before challenge of several test viruses.

tioned above, JBE-carrying cultures were remarkably (not completely) refractory to the infection of 100 m.i. of EMC virus. That is, about 10% of cell population showed degeneration. Also, preliminary experiments showed that 99% of the EMC virus disappeared from the infected tubes (cells and medium) 1 hr after inoculation ($10^{7.5}$ $TCID_{50}$ was reduced to $10^{5.5}$ $TCID_{50}$). These facts suggested that most of the EMC virus entered the cells and proceeded to the eclipse stage. If the viral process reached at least to the synthesis of viral RNA, a new interference state should be induced in the JBE-carrying cultures, according to the recent reference by Zebovits and Brown (7). Therefore, it was of interest to test whether the EMC-infected JBE-carrying cultures still had susceptibility to the other viruses as the case of JBE-carrying cultures. The JBE-carrying cultures which had been infected with 100 m.i. of EMC virus either 4 hr, or 20 days, before were used for the infectivity titrations of other viruses for comparison of JBE-carrying cells and normal KB cells. Table VI shows the results: The JBE-carrying cells infected with EMC virus 4 hr before showed definite resistance to VS virus infection only. On the contrary, JBE-carrying cells infected with EMC 20 days before had already lost the refractory state to VS virus.

Discussion. There are many reports concerning the role of interferon as a key factor in the production of interference phenomena among related or unrelated viruses. In addition, there is evidence that some viral interference is not mediated by interferon. This has been called "intrinsic interference" by Marcus and Carver (1) and as "noninterferon-mediated interference" by Zebovitz and Brown (7). Intrinsic interference is defined as a viral genome-induced cellular state of resistance to the challenge virus (1) and the replication of viral RNA by the interfering virus required to establish it (7). According to the reports (1, 7), insensitivity to dactinomycin seems to be one of the important characteristic of intrinsic interference, because the drug is known to inhibit both the formation and action of interferon in virus-injected cells (5, 8). We have confirmed that the EMC-refractory state of JBE-carrying cultures was not reversed by treatment with dactinomycin, and also that no interferon could be detected in the medium of JBE-carrying cells. The refractory state of JBE-carrying cultures was very specific to one virus (EMC) only, and had no broad spectrum of challenge virus. The resistance was strong enough to protect the cells from the severe infection of high multiplicity of EMC virus. Generally, interferon-mediated interference is not so strong as to be able to resist high multiplicity of virus infection. The several characteristics of the EMC-refractory state of JBE-carrying cells mentioned above all fit the categories of intrinsic interference. Interference with arbovirus group A (VEE,

EEE, and Sindbis) and group B (West Nile) in primary cell cultures (chick embryo and green monkey kidney) has been described (7, 1). Dubbs and Scherer (9) reported an inapparent infection of JBE virus in L-cells which was limited in duration to 21 days. Most recently while we were preparing this paper, Jarman *et al.* (10) reported the establishment of a chronic infection of West Nile virus in L-929 cells. The characteristics of West Nile-carrying L cells are somewhat different from our JBE-carrying KB cells as they show the presence of consistent morphological changes (probably incomplete CPE) and by 1 to 2 log higher production of virulent West Nile virus. The JBE-carrying KB cells cannot be differenciated from normal KB cells morphologically or by cell-growth rate, but may be recognized by the presence of virulent JBE virus in the medium and by the specific interference with EMC virus.

It is of interest that JBE-carrying cultures infected with EMC virus still had the capacity to support several viruses but not VS virus. The resistance to VS virus was temporary and disappeared in 20 days. It is therefore suggested that the VS-refractory state induced by EMC virus infection is not a permanent culture characteristic, unlike to the EMC-refractory state induced by JBE virus infection which has been continuously transferable to new generations.

In work not included in this paper it was found that a LCM-carrying KB cell line which has been keeping in this laboratory for 1 year was fully susceptible to test viruses and had no capacity to induce an EMC-refractory state after infection with JBE virus. The JBE virus released from JBE-carrying cells shows complete CPE on the LCM-carrying KB cells.

Summary. The JBE virus showed partial CPE in KB cells. Cells not subject to the CPE grew continuously and entered a virus-carrying state. The JBE-carrying KB cell line was highly resistant to superinfection with EMC virus. The refractory state seems to be an intrinsic, noninterferon-mediated, interference.

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